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Further Evidence for Stachyose and Sucrose/H^+ Antiporters on the Tonoplast of Japanese Artichoke (*Stachys sieboldii*) Tubers[1]

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Vacuoles of Japanese artichoke (*Stachys sieboldii*) tubers accumulate up to 180 mM stachyose (α-galactose-[1→6]-α-galactose-[1→6]-α-glucose-[1↔2]-β-fructose) against a concentration gradient, probably by means of an active stachyose/H^+ antiporter situated on the tonoplast. The goal of this study was to use isolated tonoplast vesicles to provide further evidence for the existence of such a transport mechanism. Therefore, vesicles were prepared from purified vacuoles of dormant tubers. ATP- and pyrophosphate (PPi)-dependent fluorescence quenching of the ΔpH probe 9-amino-6-chloro-2-methoxyacridine (ACMA) indicated that these vesicles were capable of building up a pH gradient (ΔpH, inside acid). The potent V-type H^+-ATPase inhibitor bafilomycin prevented the formation of a ΔpH in the vesicles. Bafilomycin (as well as nitrate, but not vanadate) also inhibited ATP hydrolysis, confirming the tonoplast origin of the isolated vesicles. Addition of stachyose (or sucrose, but not of mannitol) to energized vesicles caused a recovery of ACMA fluorescence, indicating a sugar-dependent dissipation of ΔpH. The rate of fluorescence recovery was dependent on the external sugar concentration used. It displayed a single saturable response to increasing sugar concentrations. Apparent K_m values of 52 and 25 mM were computed for stachyose and sucrose antiporter activities, respectively. It was also demonstrated that energized vesicles showed a much higher rate of [^{14}C]stachyose (3 mM) and [^{14}C]sucrose (1 mM) uptake than deenergized vesicles. The results obtained with isolated tonoplast vesicles were very similar to those obtained earlier with intact vacuoles and, therefore, confirm the existence of active stachyose and sucrose/H^+ antiporters on the tonoplast of *Stachys* tuber vacuoles.

Stachyose (α-Gal-[1→6]-α-Gal-[1→6]-α-Glc-[1↔2]-β-Fru) is the main storage carbohydrate of tubers of Japanese artichoke (*Stachys sieboldii*). The large central vacuoles of their parenchyma cells are the sites of stachyose storage, where up to 180 mM stachyose is accumulated against a concentration gradient (Keller and Matile, 1985). In a recent transport study with isolated *Stachys* vacuoles, the first evidence for the existence of an active stachyose carrier at the tonoplast was presented (Keller, 1992a). The observed Michaelis-Menten-type saturation kinetics, competitive inhibition by structurally related sugars, and stimulation by ATP and PPi suggested that, in *Stachys* tubers, stachyose might be accumulated in the vacuoles by a stachyose/H^+ antiport system operating on the tonoplast. In the same study, the mechanism of Suc uptake proved similar to that of stachyose.

Proton-driven antiport systems have been demonstrated in higher plants in a number of different membrane types and for both charged and uncharged solutes (Sze, 1985; Blumwald, 1987; Martinoia, 1992). In tonoplasts, two primary proton pumps have been identified: V-type ATPases and PPiases (Sze, 1985; Rae and Sanders, 1987). They are responsible for the driving of secondary transport processes including those of sugars. Tonoplast-bound Suc/H^+ antiport systems have been reported for storage tissues such as roots of red beet (Willenbrink and Doll, 1979; Getz, 1987, 1991) and sugar beet (Briskin et al., 1985; Andreev et al., 1990). However, in another Suc accumulator, sugarcane, such an antiporter seems to be lacking (Williams et al., 1990; Preisser and Komor, 1991) or its existence is uncertain (Getz et al., 1991). An equally confusing situation is found for the tonoplast-bound hexose/H^+ antiporters: their distribution in the plant kingdom and unequivocal existence is yet unresolved (for a review, see Rausch, 1991).

Considering these uncertainties and discrepancies concerning the existence of tonoplast-bound sugar/H^+ antiporters in plants as well as the uniqueness of the stachyose/H^+ antiporter recently described (Keller, 1992a), we thought it worthwhile to seek further evidence for such a system in *Stachys* tubers. Our main approach was to isolate sealed, transport-competent tonoplast vesicles via vacuoles and to follow directly the dissipation of an ATP- or PPi-fueled ΔpH across the tonoplast as a result of sugar uptake by monitoring the change of fluorescence of the optical ΔpH probe ACMA. The fluorescence-quenching strategy has been successfully used to demonstrate Suc/H^+ antiporter activities in tonoplast vesicles of sugar beet roots (Briskin et al., 1985; Andreev et al., 1990). Furthermore, a recent comparative study showed that the rates of Suc uptake by tonoplast vesicles were at least 10 times higher in vesicles isolated from vacuoles than by tissue homogenization (Getz, 1991). Therefore, the less economical but more promising way to obtain tonoplast vesicles via vacuoles was chosen.

In this report, we confirm the existence of tonoplast-bound stachyose and Suc/H^+ antiporters in *Stachys* tuber vacuoles by using isolated tonoplast vesicles, and we show that they

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Abbreviations: ACMA, 9-amino-6-chloro-2-methoxyacridine; BTP, 1,3-bis(tris-(hydroxymethyl)-methylamino)propane; F, relative fluorescence; ΔpH, transmembrane pH gradient.

display properties similar to those found by using isolated vacuoles (Keller, 1992a).

MATERIALS AND METHODS

Plant Material

Japanese artichoke (*Stachys sieboldii* Miq.) tubers imported from Rue (France) were stored in moist sand at 5°C for up to 4 months.

Special Chemicals

The macrolide antibiotic bafilomycin A_1 was purchased from Professor Altendorf, University of Osnabrück, Germany. ACMA (from Molecular Probes, Eugene, OR) was a kind gift of Alain Chanson, University of Lausanne, Switzerland. [^{14}C]Stachyose was prepared as described (Keller, 1992a) and had a specific activity of 1.57 mCi/mmol. [U-^{14}C]Suc (657 mCi/mmol) was purchased from Sigma.

Isolation of Protoplasts and Vacuoles

Protoplasts and vacuoles from the storage parenchyma of resting tubers were prepared as described (Keller and Matile, 1985; Keller, 1992a, 1992b). Briefly, the cell walls of chopped tissue were digested overnight with a combination of cellulase Y-C, pectolyase Y-23 (both enzymes from Seishin Pharmaceutical, Tokyo, Japan), and driselase (from Sigma). The resulting protoplasts were purified and selectively lysed to release the large central vacuoles intact. The vacuoles were collected by sedimentation at 1*g* and washed three times with a medium containing 0.8 M glycinebetaine, 25 mM Hepes-KOH, pH 7.6, 0.1% (w/v) BSA, and 1 mM each of $CaCl_2$, $MgSO_4$, DTT, and EGTA.

Isolation of Tonoplast Vesicles

Tonoplast vesicles were obtained by gentle homogenization of purified vacuoles in 10 volumes of tonoplast buffer (300 mM sorbitol, 2 mM Hepes-BTP, pH 7.2, 0.1% BSA, 1 mM DTT) with a glass-Teflon potter. The homogenate was centrifuged at 3000*g* for 10 min. The supernatant was centrifuged at 100,000*g* for 30 min, and the resulting pellet was suspended in tonoplast buffer and stored in 100- to 200-μL aliquots at −80°C. A tonoplast vesicle suspension of 100 μL typically contained about 30 μg of membrane protein and corresponded to about 400 μL of the original loose pellet of purified vacuoles (about 10^6 vacuoles).

ATPase Assay

ATPase activity was assayed by colorimetric measurement of Pi released from ATP. The reaction mixture (300 μL) contained (final concentrations) 50 mM BTP-Mes, pH 6.5, 3 mM $MgSO_4$, 3 mM Na_2ATP, 50 mM KCl, 5 μM gramicidin D, 0.05% (w/v) Triton X-100, and 0.1 mM Na_2MoO_4. After 60 min at 37°C, the Pi formed was determined by the method of Ames (1966).

Measurement of Proton Translocation

Proton translocation across the tonoplast was measured by monitoring the quenching and recovery of fluorescence of the ΔpH probe ACMA (Briskin, 1990; Pugin et al., 1991). ACMA was chosen as the fluorescent probe because preliminary experiments had shown that it is considerably more sensitive than the commonly used quinacrine and acridine orange (our unpublished results).

The assay mixture (650 μL) contained transport buffer (200 mM sorbitol, 20 mM Hepes-BTP, pH 7.2, 3 mM $MgSO_4$, 50 mM KCl), 0.8 μM ACMA, and 10 μL of tonoplast vesicle suspension (corresponding to about 3 μg of protein). After equilibration, the formation of a pH gradient (inside acid) was initiated by addition of 3 mM Na_2ATP (adjusted to pH 7.2 with BTP) or 0.2 mM NaPPi (final concentrations). At steady state, various concentrations of sugars were added and the change of fluorescence was monitored with a Perkin-Elmer LS-5 spectrophotofluorometer at excitation and emission wavelengths of 428 and 475 nm, respectively. Further details and additions are indicated in the text.

The recovery rate of ACMA fluorescence is expressed as ΔF/min of the linear portion of the fluorescence recovery trace, where ΔF is the recovered fluorescence relative to the difference of the fluorescence between the energized (after addition of ATP) and deenergized (after addition of monensin) steady states, respectively (Briskin, 1990).

Measurement of Sugar Uptake

Sugar uptake into tonoplast vesicles was measured by the radiolabel membrane filtration technique. The assay mixture (750 μL) contained 200 mM sorbitol, 20 mM Hepes-BTP, pH 7.2, 3 mM $MgSO_4$, 50 mM KCl, 3 mM Na_2ATP (adjusted to pH 7.2 with BTP), and 55 μL of tonoplast vesicle suspension (corresponding to about 16.5 μg of protein). After preincubation for 10 min, uptake was initiated by the addition of ^{14}C-sugar (4.5 μCi/mL) to give a final concentration of 3 and 1 mM of stachyose and Suc, respectively. At the times indicated, 135-μL aliquots were filtered through prewetted 0.45-μm cellulose nitrate membrane filters (Whatman, Maidstone, Kent, UK) on a vacuum filtration apparatus (Millipore, Wallisellen, Switzerland). The membranes were washed four times with 1 mL each of ice-cold transport buffer. The radioactivity retained by the membranes was determined by scintillation spectroscopy after they had been dissolved in 3 mL of Filter-Count (Canberra Packard, Zurich, Switzerland). Control experiments with deenergized vesicles were performed exactly as described above except that monensin (10 μM final concentration) was added 2 min prior to the addition of ^{14}C-sugars.

Protein Determination

Protein concentrations were determined by the method of Bradford (1976) using BSA as the standard.

RESULTS AND DISCUSSION

Isolation and Characterization of Tonoplast Vesicles

Tonoplast vesicles from *Stachys* tuber parenchyma were prepared directly from purified vacuoles by gentle homoge-

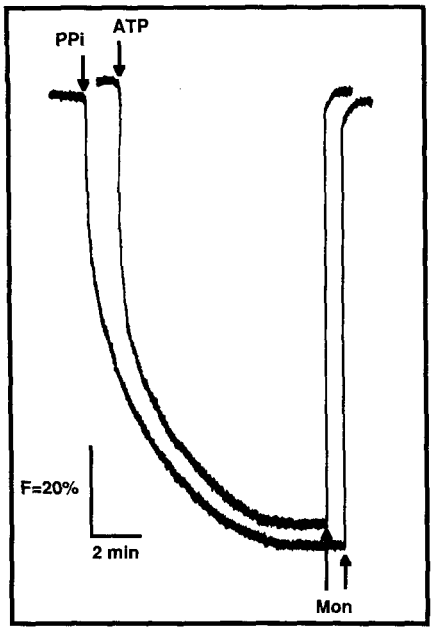

Figure 1. PPi- and ATP-dependent fluorescence quenching of ACMA in tonoplast vesicles isolated from *Stachys* tuber vacuoles. Assays were conducted as described in "Materials and Methods." The assay mixture contained 200 mM sorbitol, 20 mM Hepes-BTP, pH 7.2, 3 mM $MgSO_4$, 50 mM KCl, 0.8 μM ACMA, and about 3 μg of membrane protein. Concentrations of ATP and PPi were 3 mM and 0.2 mM, respectively. Monensin (Mon) was added at 10 μM final concentration.

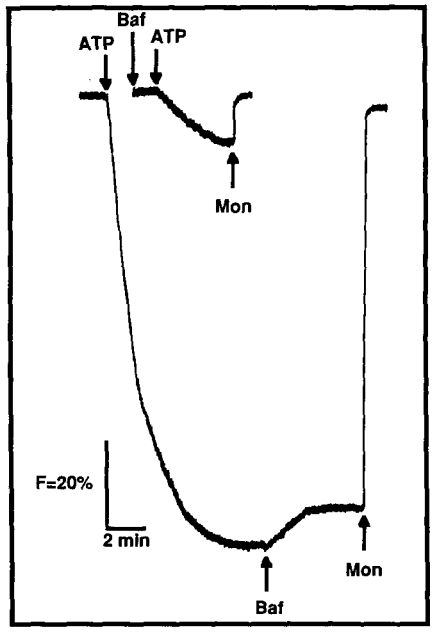

Figure 2. Effect of bafilomycin on ATP-dependent fluorescence quenching of ACMA in tonoplast vesicles isolated from *Stachys* tuber vacuoles. The assay conditions were as described in Figure 1. Ten microliters of vesicles correspond to 3 μg of membrane protein. Bafilomycin (Baf) and Monensin (Mon) were added at 12.5 nM and 10 μM final concentrations, respectively.

nization and ultracentrifugal sedimentation. When incubated with ATP or PPi, the tonoplast vesicles showed a distinct quenching of fluorescence of the ΔpH probe ACMA (Fig. 1). Such a quenching is indicative of the buildup of a ΔpH (inside acid) across the tonoplast. ACMA is a somewhat hydrophobic weak base (pK_a = 8.6) that freely permeates through membranes and accumulates in relatively acid compartments by ion trapping of the protonated form. Accumulation of ACMA in the vesicles causes its fluorescence intensity to decrease as a function of ΔpH (inside acid) (Casadio, 1991; Pugin et al., 1991).

The ΔpH generated by the proton-pumping activity of ATPase was very similar to that of PPiase (Fig. 1) and the steady state persisted almost unchanged for quite some time (typically for at least 30 min). The ΔpH at steady state represents mainly a balance between enzymic proton pumping and proton leakage at the thermodynamically possible level. The ionophore monensin completely dissipated the ΔpH.

The potent V-type H^+-ATPase inhibitor bafilomycin A_1 (Bowman et al., 1988) largely prevented the formation of a ΔpH when applied prior to ATP addition at a concentration of 12.5 nM (Fig. 2, upper trace). Higher bafilomycin concentrations (20–30 nM) completely inhibited the H^+-pumping activity of the ATPase (not shown). Such a concentration-dependent inhibition is good evidence that the isolated vesicles were indeed of tonoplast origin. When 12.5 nM bafilomycin was applied to energized vesicles, a slight initial dissipation of ΔpH was often observed, whereupon a new steady state was reached (Fig. 2, lower trace). When higher bafilomycin concentrations (20–30 nM) were applied to energized vesicles, the ΔpH dissipated relatively quickly (not shown), most probably as a result of the complete shutting off of the proton pump and the subsequent proton leakage and/or unknown deleterious effects on membrane integrity by bafilomycin. For our purposes, we were endeavoring to find a bafilomycin concentration that would diminish proton pumping without completely dissipating the ΔpH, and, therefore, 12.5 nM was chosen (Fig. 2).

The tonoplast origin of the isolated vesicles was further tested by comparison of the effect of inhibitors on their ATPase activity. Table I shows that the vacuolar ATPase

Table I. *Effect of inhibitors on ATPase activity associated with tonoplast vesicles isolated from Stachys tuber vacuoles*

Assays were conducted as described in "Materials and Methods." The assay mixture contained 50 mM BTP-Mes, pH 6.5, 3 mM $MgSO_4$, 3 mM Na_2ATP, 50 mM KCl, 5 μM gramicidin D, 0.05% (w/v) Triton X-100, 0.1 mM Na_2MoO_4, and about 3 μg of membrane protein. Values are means ± SE of four determinations of a pooled tonoplast vesicle fraction originating from four separate vesicle isolations.

Inhibitor	Concentration	ATPase Activity	
		μmol Pi mg^{-1} protein h^{-1}	%
Control		12.2 ± 0.8	100
Na_3VO_4	0.1 mM	12.8 ± 0.8	105
KNO_3	50 mM	3.2 ± 1.7	26.5
Bafilomycin	20 nM	3.7 ± 1.8	30.6

inhibitors nitrate and bafilomycin both inhibited ATP hydrolysis by about 70%, whereas the plasmalemma ATPase inhibitor vanadate did not inhibit hydrolysis. These results and the fact that the original vacuoles used for vesicle preparations were quite pure (less than 10% of extravacuolar contamination, see Keller and Matile, 1985; Keller, 1992a, 1992b) both indicate that our membrane preparations were highly enriched in tonoplast vesicles. Furthermore, they were capable of generating and maintaining a ΔpH (see above) and contained the commonly found Ca^{2+}/H^+ antiporter (Blumwald and Poole, 1986; Bush and Sze, 1986; Chanson, 1991; Peisser and Komor, 1991) as revealed by a rapid recovery of ACMA fluorescence when 1 mM $CaCl_2$ was added to energized vesicles (not shown). Our tonoplast vesicle preparations, therefore, showed enough features to allow the elucidation of putative sugar/H^+ antiport systems.

Sugar/H^+ Antiport

Addition of stachyose (6.25 and 50 mM external concentrations) to ATP-energized vesicles caused a recovery of ACMA fluorescence (Fig. 3), indicating a sugar-dependent dissipation of the original ΔpH and, possibly, reflecting the activity of a stachyose/H^+ antiport system recently found on the tonoplast (Keller, 1992a). As a control, the effect of the addition of the nontransportable sugar alcohol mannitol (Keller, 1992a) at 0, 50, or 100 mM final concentration to energized vesicles was tested and did not show any change of ACMA fluorescence (not shown). The rate of stachyose-induced fluorescence recovery was obviously dependent on the sugar concentrations used (Fig. 3). When the stachyose-dependent rates of fluorescence recovery were assayed as a function of increasing stachyose concentrations (6.25–75 mM), Michaelis-Menten-type saturation kinetics were observed (Fig. 4).

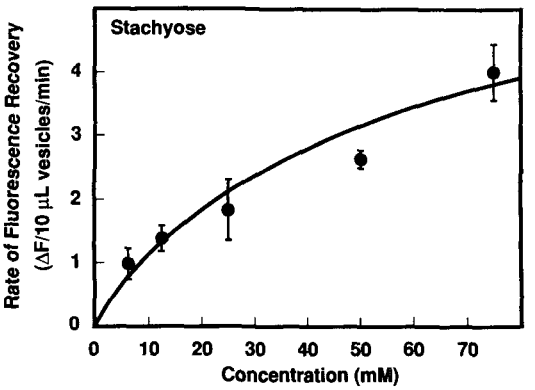

Figure 4. Concentration dependence of stachyose-induced rate of ACMA fluorescence recovery in tonoplast vesicles isolated from *Stachys* tuber vacuoles and energized by ATP. The assay conditions were as described in Figure 1. Ten microliters of vesicles correspond to 3 μg of membrane protein. Values are means ± SE of five separate experiments.

Addition of Suc (6.25 and 50 mM external concentrations) to ATP-energized vesicles caused a recovery of ACMA fluorescence similar to that observed with stachyose (Fig. 5). Likewise, the concentration dependence of the Suc-induced rate of fluorescence recovery showed saturation kinetics (Fig. 6).

Lineweaver-Burk computations of the kinetic data presented in Figures 4 and 6, applying the weighted regression

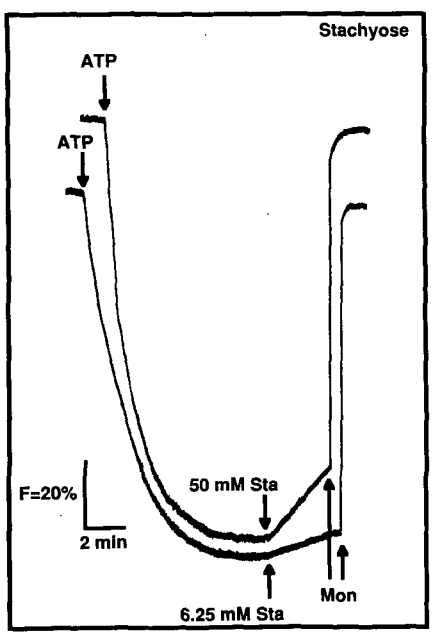

Figure 3. Effect of stachyose on the rate of ACMA fluorescence recovery in tonoplast vesicles isolated from *Stachys* tuber vacuoles and energized by ATP. The assay conditions were as described in Figure 1. Sta, Stachyose.

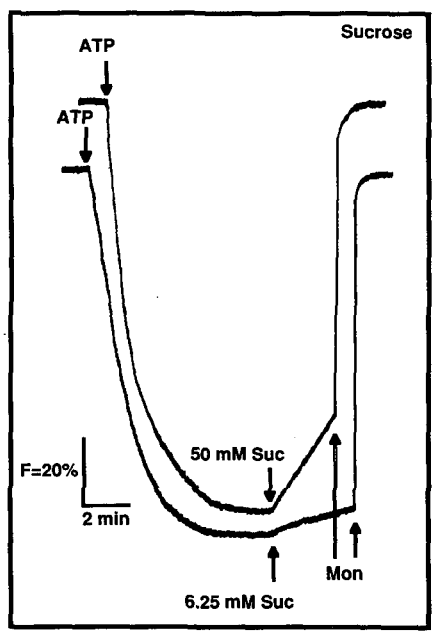

Figure 5. Effect of Suc on the rate of ACMA fluorescence recovery in tonoplast vesicles isolated from *Stachys* tuber vacuoles and energized by ATP. The assay conditions were as described in Figure 1.

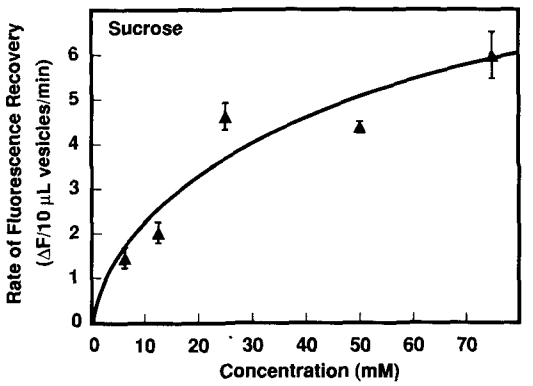

Figure 6. Concentration dependence of Suc-induced rate of ACMA fluorescence recovery in tonoplast vesicles isolated from *Stachys* tuber vacuoles and energized by ATP. The assay conditions were as described in Figure 1. Ten microliters of vesicles correspond to 3 μg of membrane protein. Values are means ± SE of four separate experiments.

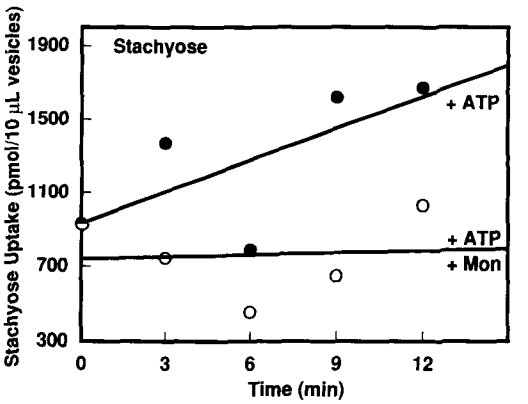

Figure 7. Time course of [^{14}C]stachyose uptake by tonoplast vesicles isolated from *Stachys* tuber vacuoles. The assay mixtures contained 200 mM sorbitol, 20 mM Hepes-BTP, pH 7.2, 3 mM $MgSO_4$, 50 mM KCl, 3 mM ATP and 3 mM [^{14}C]stachyose. The vesicles were preincubated (energized) for 10 min prior to the addition of [^{14}C]-stachyose. Monensin (Mon, 10 μM) was added to the controls, after preincubation with ATP, 2 min prior to the addition of [^{14}C]stachyose to deenergize the vesicles. Ten microliters of vesicles correspond to 3 μg of membrane protein. Values are from a single representative experiment of a total of three experiments.

method of Wilkinson (1961), revealed apparent K_m values of 52 and 25 mM and V_{max} values of 6.3 and 7.7 ΔF (10 μL vesicles)$^{-1}$ min^{-1} for the stachyose- and Suc-dependent rates of ACMA fluorescence recovery, respectively. It is interesting to note that these K_m values obtained by using the present tonoplast vesicle/fluorescence quenching approach are surprisingly similar to those obtained earlier by using the intact vacuole/^{14}C-sugar uptake approach (K_m values of 53 and 32 mM for stachyose and Suc, respectively [Keller, 1992a]). In the former approach, the sugar-dependent ΔpH is measured, whereas in the latter approach the actual sugar uptake is measured. The observed similarities strongly suggest that, with both approaches, the same carriers were investigated and that they are of the sugar/H$^+$ antiporter type.

Application of specific V-type H$^+$ATPase inhibitors (such as nitrate or bafilomycin) to energized, leak-tight vesicles should enhance the subsequent sugar-dependent rate of ACMA fluorescence recovery because it prevents the ATPase-driven recycling of protons exported by the sugar/H$^+$ antiporters. The use of nitrate (20–50 mM KNO_3) proved to be impossible because it caused the ATP-induced ACMA fluorescence quenching to revert rapidly (not shown), which might be related to nitrate's ability to form a lipophilic ion pair with protonated ACMA, thus artifactually dissipating the ΔpH (Pugin et al., 1991). Therefore, the inhibitor of choice in our experimental system was bafilomycin, which had been shown, at 12.5 nM, to inhibit proton-pumping substantially without much effect on the ΔpH when applied to energized vesicles (Fig. 2). Table II shows that 12.5 nM bafilomycin roughly doubled the rate of ACMA fluorescence recovery induced by addition of 25 mM of both stachyose and Suc. This enhancement is in full agreement with the sugar/H$^+$ antiport concept presented here.

Finally, additional evidence for sugar/antiport activity of the isolated vesicles was obtained from experiments using ^{14}C-labeled substrates. When energized vesicles were incubated with [^{14}C]stachyose (3 mM) and [^{14}C]Suc (1 mM), respectively, time-dependent uptake was clearly apparent

Table II. *Effect of bafilomycin on the stachyose- and Suc-dependent rate of ACMA fluorescence recovery in tonoplast vesicles isolated from Stachys tuber vacuoles energized by ATP*

Assays were conducted as described in "Materials and Methods." The assay mixture contained 200 mM sorbitol, 20 mM Hepes-BTP, pH 7.2, 3 mM $MgSO_4$, 50 mM KCl, 0.8 μM ACMA, and about 3 μg of membrane protein (10 μL vesicles). The vesicles were energized by addition of 3 mM ATP. After steady state occurred, 12.5 nM bafilomycin was added to inhibit ATPase activity. After a second steady state was reached, 25 mM stachyose or Suc was added and the recovery rate of fluorescence quenching of ACMA was monitored.

Treatment	Rate of Fluorescence Recovery			
	Stachyose		Suc	
	Exp. 1	Exp. 2	Exp. 1	Exp. 2
	ΔF (10 μL vesicles)$^{-1}$ min^{-1}		ΔF (10 μL vesicles)$^{-1}$ min^{-1}	
Control	2.56 (100%)	1.59 (100%)	4.09 (100%)	3.94 (100%)
+Bafilomycin	4.86 (190%)	2.82 (177%)	8.18 (200%)	9.15 (232%)

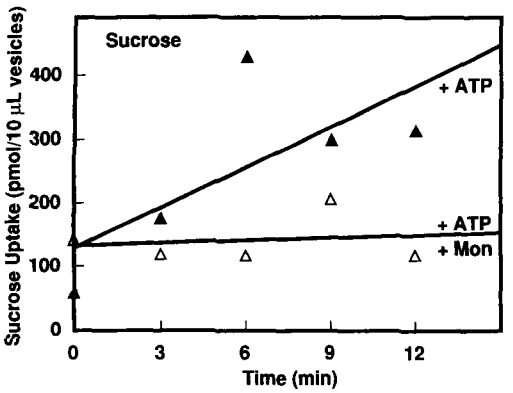

Figure 8. Time course of [^{14}C]Suc uptake by tonoplast vesicles isolated from *Stachys* tuber vacuoles. The assay mixtures contained 200 mM sorbitol, 20 mM Hepes-BTP, pH 7.2, 3 mM $MgSO_4$, 50 mM KCl, 3 mM ATP, and 1 mM [^{14}C]Suc. The vesicles were preincubated (energized) for 10 min prior to the addition of [^{14}C]Suc. Monensin (Mon, 10 μM) was added to the controls, after preincubation with ATP, 2 min prior to the addition of [^{14}C]Suc to deenergize the vesicles. Ten microliters of vesicles correspond to 3 μg of membrane protein. Values are from a single representative experiment of a total of five experiments.

(Figs. 7 and 8). For both sugars, the uptake rates were substantially higher with energized than with deenergized vesicles, indicating ΔpH-dependent uptake.

CONCLUSION

When tonoplast vesicles were prepared directly from purified *Stachys* tuber vacuoles, they proved to be of high quality as judged by their ability (a) to generate and maintain a ΔpH when incubated with ATP or PPi and (b) to dissipate a ΔpH in the presence of stachyose and Suc. Furthermore, the sugar-induced rates of ΔpH dissipation displayed Michaelis-Menten-type saturation kinetics. These results, when considered with the results of the ^{14}C-sugar uptake experiments with vesicles (this study) and isolated vacuoles (Keller, 1992a), provide strong evidence that, in *Stachys* tubers, stachyose and Suc are transported by a sugar/H^+ antiport mechanism operating on the tonoplast. However, they are not helpful in determining if these substrates are transported by the same or different antiporters. The stage is now set to perform additional experiments, such as detailed inhibitor studies, to characterize further the antiporter(s). These will be performed by using our tonoplast vesicles for comparative uptake studies with ^{14}C-labeled sugars.

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